Mechanistic alternatives in phosphate monoester hydrolysis: what conclusions can be drawn from available experimental data?

Johan Åqvist[1], Karin Kolmodin[1], Jan Florian[2] and Arieh Warshel[2]

Phosphate monoester hydrolysis reactions in enzymes and solution are often discussed in terms of whether the reaction pathway is associative or dissociative. Although experimental results for solution reactions have usually been considered as evidence for the second alternative, a closer thermodynamic analysis of observed linear free energy relationships shows that experimental information is consistent with the associative, concerted and dissociative alternatives.

Addresses: [1]Department of Molecular Biology, Uppsala University, Biomedical Center, Box 590, S-75124 Uppsala, Sweden. [2]Department of Chemistry, University of Southern California, Los Angeles, CA 90098-1062, USA.

Correspondence: Johan Åqvist
E-mail: aqvist@xray.bmc.uu.se

Chemistry & Biology March 1999, **6**:R71–R80
http://biomednet.com/elecref/10745521006R0071

 ISSN 1074-5521

The hydrolysis of phosphate esters is an important reaction in many biological systems. Over the past forty years a considerable amount of work has been devoted to the mechanisms of phosphate ester hydrolysis and related phosphoryl transfer reactions in aqueous solution; one of the main objectives has been to distinguish between the different mechanistic pathways corresponding to associative and dissociative phosphoryl transfer processes. The extremes of these two mechanisms involve expulsion of a monomeric metaphosphate ion prior to nucleophilic addition for the dissociative pathway (unimolecular elimination), and formation of a pentacoordinated transition state or transient intermediate prior to leaving group expulsion for the associative mechanism (bimolecular association).

The currently dominating view regarding phosphate ester hydrolysis in solution is that diesters and triesters follow a more associative-like pathway, whereas the hydrolysis of monoesters proceeds by a dissociative mechanism [1–4]. The latter does not necessarily mean that free metaphosphate is formed as an intermediate, but it is rather interpreted such that the rate-limiting transition state (TS) mainly involves leaving-group bond fission with little bond formation to the incoming nucleophile. It would then be expected that the total axial phosphorous–oxygen bond order is lower in the TS than in the reactants and products. This dogma is, however, repeatedly challenged by enzyme studies in which phosphate monoester cleavage is often interpreted in terms of more or less associative bimolecular mechanisms [5–12]. The other alternative, namely that enzymic monoester cleavage generally proceeds by a dissociative unimolecular elimination mechanism as in solution, has, however, also found its proponents [4,13]. It is also possible that the energetics of the associative and dissociative mechanisms are similar in solution and that enzymes catalyzing phosphate monoester (P–O bond) cleavage actually can alter the mechanism of their uncatalyzed reaction counterparts.

This controversy can be examined further by a closer thermodynamic analysis of available experimental data that have been taken as evidence for the dissociative mechanism of phosphate monoester hydrolysis in solution. It appears that most, if not all, experimental information about the nature of phosphate hydrolysis in solution leads to much less conclusive interpretations than previously thought. In fact, the evidence invoked for postulating a dissociative mechanism is found to be equally consistent with an associative or concerted mechanism.

Phosphate ester hydrolysis rates

The original unimolecular dissociative hydrolysis mechanism for simple phosphate monoesters (e.g. $CH_3OPO_3^{2-}$) was proposed as an explanation for the higher reactivity of the (singly protonated) monoanion than that of the neutral and dianionic species [14,15]. Furthermore, the possibility of a bimolecular displacement through OH^- attack on the uncharged (doubly protonated) phosphate group was considered improbable because monoesters have a significantly higher reactivity than diesters and triesters. In other words, if the reaction:

$$H_2O + ROPO_3H^- \xrightarrow{k} HOPO_3H^- + ROH \qquad (1)$$

were to involve a pre-equilibrium proton transfer (PT) step followed by OH^- attack:

$$\begin{aligned} &H_2O + ROPO_3H^- \overset{K_{eq}}{=\!=} OH^- + \\ &ROPO_3H_2 \xrightarrow{k'} HOPO_3H^- + ROH \end{aligned} \qquad (2)$$

then the overall hydrolysis rate (k) would be determined by the pK_a difference involved in the pre-equilibrium step (ΔG_{PT} ~19–20 kcal/mol) together with the rate for OH^- attack (k′) on the neutral ester. The latter rate was then assumed to be the same as that for OH^- attack on the diester anion and triesters (~$10^{-2}s^{-1}M^{-1}$) [15,16], which is clearly too slow to account for the observed value of k. An alternative mechanism for monoester monoanion hydrolysis was therefore proposed:

$$\begin{aligned} &H_2O + ROPO_3H^- \rightarrow H_2O + RO(\overset{+}{H})PO_3^{2-} \rightarrow \\ &H_2O + PO_3^- + ROH \rightarrow HOPO_3H^- + ROH \end{aligned} \qquad (3)$$

where protonation of the leaving group could also be accomplished through intervening solvent molecules.

Recent theoretical calculations (*ab initio* plus solvation) [17] addressing the assumption that the rate of OH^- attack on a triester (trimethylphosphate) is similar to that on the corresponding neutral monoester ($CH_3OPO_3H_2$) showed that the barrier for hydroxide ion addition to the monoester is 12–13 kcal/mol lower than for the triester, making a pre-equilibrium PT mechanism according to equation 2 quite feasible. The reduced barrier for the monoester was found to arise from hydrogen-bonding interactions and differential solvation of the two esters. Furthermore, in the absence of any direct experiment about the energetics for the attack of OH^- on a neutral monoester, it appears much more reasonable to accept the results of reliable *ab initio* studies than the traditional estimate of this energy.

In view of these results, is there any compelling evidence, based on overall reaction rates, that rules out bimolecular displacement through an associative mechanism? Here, we will consider mainly the monoester dianion reaction because the dianion is the substrate in many enzyme reactions, including those involving G proteins and several phosphatases. By analogy with the monoanion, this reaction has also been postulated to proceed through a dissociative unimolecular elimination pathway [3,4,16]. The mechanisms corresponding to equations 2 and 3 for phosphate monoester dianion hydrolysis are:

$$H_2O + ROPO_3^{2-} \overset{K_{eq}}{\rightleftharpoons} OH^- + ROPO_3H^- \overset{k'}{\rightarrow} HOPO_3^{2-} + ROH \quad (4)$$

and

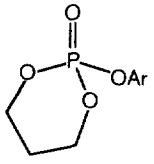
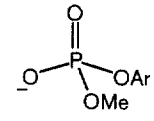
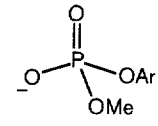
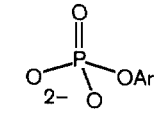
$$H_2O + ROPO_3^{2-} \rightarrow H_2O + PO_3^- + RO^- \rightarrow HOPO_3^{2-} + ROH \quad (5)$$

for the associative mechanism with pre-equilibrium PT and for the dissociative mechanism, respectively.

Experimental results, from studies carried out by Kirby and Varvoglis [16,18] in the late 1960s, can be used to illustrate that the difference in rate between the monoanion and dianion reactions is fully consistent with the mechanism of equation 4. The experimental results for 2,4-dinitrophenyl phosphate esters [18] are summarized in terms of standard free energies of activation in Table 1. By comparing the barrier for OH^- attack on the triester with that on the diester anion a reasonable estimate of the pure electrostatic (repulsive) effect can be obtained because no hydrogen bonding to the nucleophile is possible for the unprotonated phosphate groups. This gives $\Delta\Delta G^{\ddagger}(Q_{phos} = 0 \rightarrow Q_{phos} = -1)$ ~+5 kcal/mol associated with (mainly) changing the charge state of the substrate from neutral to −1. This estimate might be complicated by a possible rate acceleration caused by the ring, which would make the electrostatic effect even smaller. The magnitude of the repulsive electrostatic effect is found to be quite small. If we consider the mechanism of equation 2 for the third reaction in Table 1 (diester hydrolysis), we obtain $\Delta G^{\ddagger}(k') = 30 - 23 = 7$ kcal/mol for attack of OH^- on the neutral protonated diester. This estimate is obtained by subtracting from the overall activation barrier the free energy of the pre-equilibrium step, $\Delta G^0_{eq} = 23$ kcal/mol, obtained using a pKa of −1.16 for methyl 2,4-dinitrophenyl phosphate [19].

Table 1

Activation barriers for 2,4-dinitrophenyl phosphate reactions.

Reaction	$\Delta G^{\ddagger}$ (kcal/mol)
OH^- + 	18.2
OH^- + 	23.0
H_2O + 	30.0
H_2O + 	26.0

Calculated from second-order rate constants in Table 5 of [18] and converted to standard state free energies (25°C, 1 M) using Eyring's equation with a transmission factor of unity.

Considering the energetics of the fourth reaction in Table 1, namely dianion hydrolysis, according to the mechanism of equation 4, the pre-equilibrium step gives $\Delta G^0_{eq} = 15$ kcal/mol using the second $pK_a = 4.5$ of 2,4-dinitrophenyl phosphate [19]. A reasonable estimate of the barrier for OH^- attack on the monoanion, which now has one proton that could stabilize the incoming nucleophile, is obtained by adding the 'pure' electrostatic repulsion effect of +5 kcal/mol to the barrier (according to equation 2) for OH^- attack on the neutral protonated diester. This yields $\Delta G^{\ddagger}(k') = 7 + 5 = 12$ kcal/mol for addition of OH^- to the monoester monoanion. So the overall reaction barrier of the dianion is predicted to be $\Delta G^{\ddagger} = 15 + 12 = 27$ kcal/mol, which is in good agreement with the experimentally observed free energy barrier of ~26 kcal/mol (Table 1). The experimental results for these compounds, which have often been quoted as evidence for a dissociative mechanism [2,4,14,15,20], are therefore entirely consistent with an associative process. The observed hydrolysis rates alone therefore do not provide sufficient evidence for a unimolecular

elimination mechanism in the reactions of phosphate monoesters. Of course, the reactions may still involve such a dissociative mechanism but this cannot be proven simply by considering the observed rate constants and, as will be seen below, the interpretation of other experimental facts is also subject to ambiguities.

What is the evidence for dissociative pathways?

Most of the literature on phosphomonoester hydrolysis has favoured the dissociative or metaphosphate mechanism [1–4,20]. Apart from the arguments based on overall rate comparisons for monoesters, diesters and triesters that do not seem to hold, the main lines of evidence quoted are: linear free energy relationships (LFERs) indicating a strong dependence of the rate on the leaving group; a large ^{18}O isotope effect for the leaving group bridging oxygen; and activation entropies close to zero. We will examine further each of these types of experiments.

Linear free energy relationships

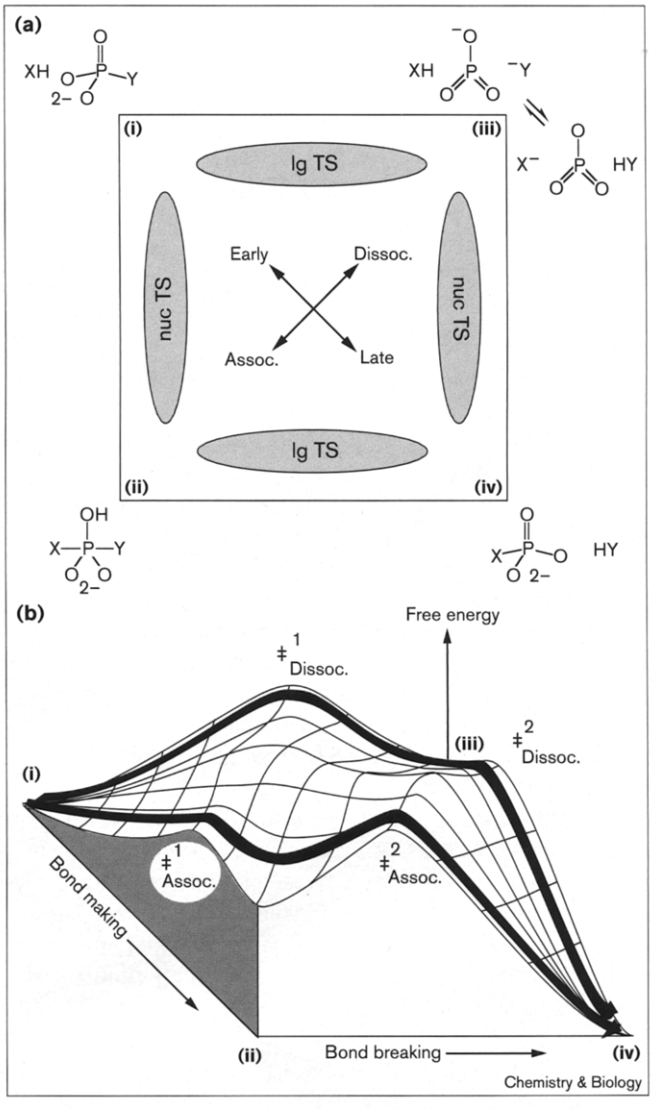
LFERs between the rates of phosphoester bond cleavage and the pK_a values of the nucleophile (nuc) and leaving group (lg) have been used frequently in the analysis of these reaction mechanisms. The corresponding Brønsted coefficients (β_{nuc} and β_{lg}) are defined by:

$$\beta_{nuc/lg} = \frac{d\log k}{dpK_a^{nuc/lg}} = -\frac{\Delta\Delta G^{\ddagger}_{tot}}{1.36\Delta pK_a^{nuc/lg}} \qquad (6)$$

where $\Delta\Delta G_{tot}^{\ddagger}$ denotes the overall (rate-limiting) activation free energy barrier of the reaction. A schematic reaction with the metaphosphate and pentacoordinate phosphate group as limiting mechanisms is shown in Figure 1a for the phosphomonoester dianion (equations 4,5). The actual topography of the solution energy surface has not been fully established. A valley, or rather the existence of minima, along the associative–dissociative diagonal has been implicated from both experimental data [19] and early theoretical calculations [7,21,22]. More recent *ab initio* Langevin dipoles studies have provided a more complex picture with activation barriers along the associative and dissociative pathways [23,24]. Furthermore, we have carried out semi-empirical calculations with geometry optimization in a high-dielectric continuum on the $CH_3OP(O_3H)OCH_3^{2-}$ structure utilizing the AM1-SM2 and PM3-SM3 hamiltonians [25]. Both of these models locate associative-like minima with slightly increased axial bond order to P compared with the methyl phosphate dianion. A local minimum somewhere along such a valley will correspond to a very high-energy structure that would not be easy to observe experimentally. The general features of the potential surface can be outlined as in Figure 1.

As indicated in Figure 1a, we have several transition states on the free energy surface for phosphate hydrolysis [23], whereas traditional interpretations of LFERs have assumed a single transition state for each of the pathways

Figure 1



(a) Schematic reaction coordinate diagram for phosphoryl transfer between two axial groups X and Y; (i) reactants, (ii) associative transient intermediate, (iii) dissociative transient intermediate and (iv) products. **(b)** Schematic free energy surface for the monoester dianion phosphoryl transfer reaction between X and Y; labelling of the corners of the diagram as in (a).

(associative and dissociative). When examining LFERs one has to consider the effect of the free energy difference in each step on the corresponding activation barriers [26–28]. Thus, changing the pK_a (or nucleophilicity) of the nucleophile or leaving group does not only affect the energetics of steps involving proton transfer with that group, but also affects the overall reaction free energy as well as the pK_a of the attached phosphate group. The effect of the leaving group on the reaction free energy, for the reaction $XO\text{–}PO_3^{2-} + H_2O \rightleftharpoons HO\text{–}PO_3^{2-} + XOH$ is given by an empirical equation from Bourne and

Williams [29] (in kcal/mol, after correction for the 55 M concentration of water):

$$\Delta G^{o}_{rxn} \cong 0.5 pK_a^{XOH} - 7.8 \quad (7)$$

Furthermore, the effect of changing the pK_a of XOH when attached to the phosphate group causes the pK_a values of the phosphate to shift as ΔpK_a (phosphate)/ΔpK_a(lg) ~0.23 (this value is calculated from the data in [19,29]).

A more detailed energetic picture (Figure 2) helps to relate shifts in nucleophile and leaving group pK_a values to changes in activation barriers. This figure depicts three sections of the overall energy surface: one (Figure 2a,b) along the associative corners (i → ii → iv: pre-equilibrium PT), one (Figure 2c,d) along the dissociative corners (i → iii → iv: unimolecular elimination) and one (Figure 2 e,f) along a concerted pathway (i → iv). These sections are drawn as intersections of parabolas (including those representing proton transfers) to clarify the relationship to Marcus-type LFER treatments [26–28], where the transition-state energies are correlated with the crossing of the relevant parabolas. In the associative case the proton transfers are depicted as stepwise, with distinct resonance structures, to indicate the effects of pK_a shifts on the energy of each state. Although such proton transfers might in reality be concerted with bond making/breaking to P, this would simply reflect mixing of the relevant resonance structures. In the dissociative case, it is not clear for the dianion reaction exactly how proton transfer from the nucleophile to the leaving group would proceed (e.g. solvent assisted or via transient protonation of metaphosphate), but the pK_a difference between the two groups will inevitably be involved in the energetics as illustrated by the two high-energy resonance forms of Figure 2c,d.

In analyzing the LFERs we will try to draw quantitative conclusions using Marcus' relationship for successive steps. That is, for steps that involve crossing between two adjacent resonance structures, i → j, we can use:

$$\beta_{i\to j} = \frac{d(\Delta G)^{\ddagger}_{i\to j}}{d(\Delta G^{0}_{i\to j})} = \frac{d[(\Delta G^{0}_{i\to j} + \lambda_{i\to j})^2 / 4\lambda_{i\to j}]}{d(\Delta G^{0}_{i\to j})} = \frac{\Delta G^{0}_{i\to j}}{2\lambda} + \frac{1}{2} \quad (8)$$

where λ is the reorganization free energy. Assuming that λ is significantly larger than ΔG^{o} we obtain:

$$\beta_{i\to j} = \frac{\Delta\Delta G^{\ddagger}}{\Delta\Delta G^{o}} \approx \frac{1}{2} \quad (9)$$

To obtain the LFER for each mechanism considered in Figure 2 we have to express $\Delta\Delta G^{o}$ for all resonance structures in terms of ΔpK_a^{nuc} or ΔpK_a^{lg}.

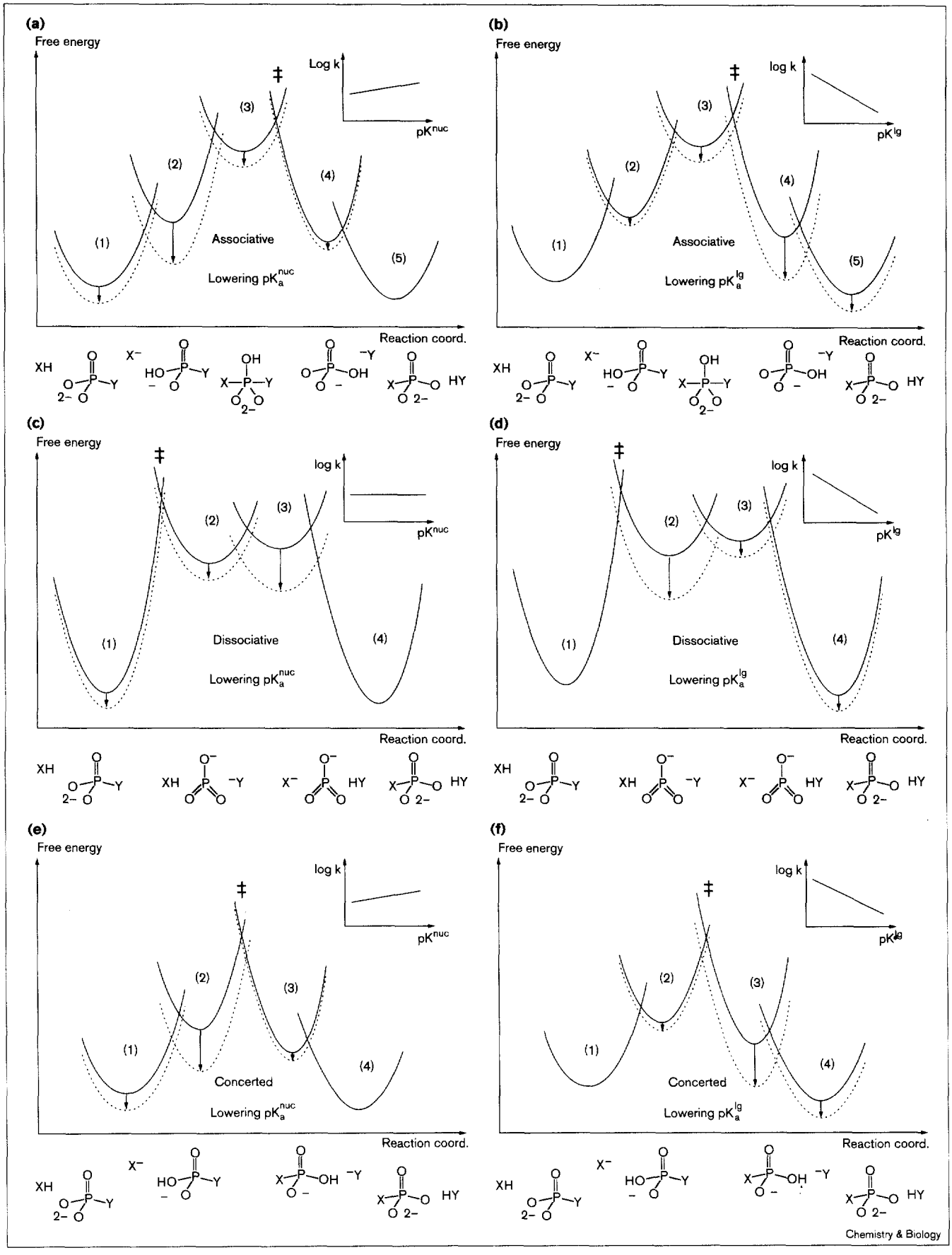
First, we consider the associative reaction, which is rate limited by cleavage of the bond to the leaving group (late TS, see Figure 2a). Here, the ΔG^{o} changes due to lowering the pK_a of the nucleophile are drawn relative to the energy of the product. The reactant state (1) is reduced by $0.5\Delta pK_a^{nuc}$ kcal/mol (equation 7). Because the free energy difference between (2) and (1) is determined by the pK_a difference between the phosphate group and that of the nucleophile, the energy of state (2) is reduced by $(1.36 + 0.5)\Delta pK_a^{nuc}$. The energy of state (4) will also drop relative to the product (5) because, in this case, pK_a^{phos} will change by $0.23\Delta pK_a^{nuc}$ [19,29], yielding a free energy shift in kcal/mol of $1.36 \times 0.23\Delta pK_a^{nuc} = 0.3\Delta pK_a^{nuc}$. It is less clear exactly how much the high-energy state (3) would be shifted, but its energy is also likely to be somewhat lowered relative to the product (but not as much as state 2) because the nucleophilicity of X decreases and its bonding to P will be weaker in the pentacoordinated complex. A small positive value of Brønsted coefficient β_{nuc} will result, because the main effect of lowering the nucleophile pK_a is exerted on the energy of state (2), which is not rate determining if the TS is late.

Figure 2b shows the corresponding effects for an associative mechanism if instead the pK_a of the leaving group is lowered. Now the main effect is found on state (4), which is directly involved in the curve crossing that determines the barrier for a late TS. The free energy of state (4) is lowered by $(1.36 + 0.5)\Delta pK_a^{lg}$ relative to the reactant state (1). In the extreme case corresponding to a lowering of the high-energy state (3) by the same amount as (4), the rate limiting TS determined by the crossing of these two curves will also be subjected to the same shift. This would then yield a maximum negative value of $\beta_{lg} = -(1.36 + 0.5)/1.36 = -1.4$ because the entire lowering of state (3) is realized as a reduction of the activation barrier relative to the reactants. In the other extreme case in which state (3) is unaffected by lowering pK_a^{lg}, equation 9 predicts a value of $\beta_{lg} = -0.7$. It is therefore clear that a large negative value of β_{lg} will be observed.

For the dissociative mechanism, Figure 2c,d demonstrates the effects of nucleophile and leaving-group pK_a values on the energetics of phosphate transfer. It is worth noting here that for a completely dissociative mechanism (free metaphosphate anion as an intermediate) with an early TS the value of β_{nuc} will be exactly zero, as states (1) and (2) are shifted by the same amount. On the other hand, β_{lg} will again be negative as can be seen from Figure 2d. As the

Figure 2

Free-energy diagram depicting the different (diabatic) states **(a,b)** along a stepwise associative phosphoryl transfer pathway involving pre-equilibrium proton transfer to the phosphate group, **(c,d)** along a stepwise dissociative phosphoryl transfer pathway involving unimolecular elimination of metaphosphate from the dianion, and **(e,f)** along a concerted pathway also involving pre-equilibrium proton transfer. The reaction in (a–d) is considered to be rate-limited by leaving-group bond fission. The dashed curves show how the free energies of the different states are shifted when the pK_a of the nucleophile (a,c,e) and the leaving group (b,d,f) is lowered (see text for details).


(a)
Free energy
Log k
pK^nuc
(1)
(2)
(3)
(4)
(5)
Associative
Lowering pK_a^nuc
Reaction coord.
(b)
Free energy
log k
pK^lg
Associative
Lowering pK_a^lg
Reaction coord.
(c)
Free energy
log k
pK^nuc
Dissociative
Lowering pK_a^nuc
Reaction coord.
(d)
Free energy
log k
pK^lg
Dissociative
Lowering pK_a^lg
Reaction coord.
(e)
Free energy
log k
pK^nuc
Concerted
Lowering pK_a^nuc
Reaction coord.
(f)
Free energy
log k
pK^lg
Concerted
Lowering pK_a^lg
Reaction coord.
Chemistry & Biology

early TS is determined only by the crossing of states (1) and (2), however, the Marcus type of relationship (equation 9) between $\Delta G^{\ddagger}$ and ΔG^{o} for that step can again be used to predict the value of β_{lg}. As equation 7 gives the lowering of the product state (4) relative to the reactants (1) as $0.5\Delta pK_a^{lg}$, and as the energy difference between states (3) and (4) is unaffected by the leaving group, state (3) will also be lowered by $0.5\Delta pK_a^{lg}$. The free energy difference between states (3) and (2) is given by $1.36\Delta pK_a^{lg}$ so state (2) changes by $(1.36 + 0.5)\Delta pK_a^{lg}$. Applying equation 9 to the crossing of states (1) and (2), a value of $\beta_{lg} = 0.9/1.36 = -0.7$ is obtained. It therefore appears more difficult to rationalize observed values around or smaller than −1 than with the pre-equilibrium PT mechanism, where such a value can arise from a lowering of both states (3) and (4).

To analyze the phosphate monoester solvolysis data reported in [3], to check whether the experimental values of $\beta_{nuc} \sim 0.1$ and $\beta_{lg} \sim -0.9$ are mutually consistent in the framework of an associative mechanism, we use the experimental $\beta_{lg} = -0.9$ (and equation 9) to determine the shift of energy of state (3) in the associative mechanism of Figure 2b and obtain a value of $\Delta\Delta G^{0}_{1\rightarrow 3} = 0.5\Delta pK_a^{lg}$ kcal/mol. That is, state (3) is lowered by approximately the same amount as the products (or, conversely, the reactants when pK_a^{nuc} is changed). We can then predict the LFER for lowering the pKa of the nucleophile in the associative mechanism (Figure 2a). The reduction of the TS relative to the reactants becomes $\Delta\Delta G^{\ddagger}_{1\rightarrow 4} = \frac{1}{2} \times 0.2\ \Delta pK_a^{nuc}$ and the result is therefore $\beta_{nuc} = +0.1$. Hence, the associative model is again found to be in good agreement with the experiments which yield a β_{nuc} of the same magnitude.

Here it is, however, worth noting that the above analysis does not rely on any assumption regarding the detailed nature of state (3) in Figures 2a,b. The same analysis could therefore be applied with a transient intermediate having low axial bond orders to P, that is a dissociative one, as long as a pre-equilibrium PT to the phosphate group is considered. In such a case one would still observe a large negative value of β_{lg}, but for a TS that is now associated with the incoming nucleophile rather than with the leaving group. This illustrates that the protonation state of the phosphate group in the TS is an equally important issue as its bond orders to the axial ligands.

Figure 1b shows two reaction paths that would yield a larger absolute value of β_{lg} than β_{nuc}. In fact, as long as proton transfer from the nucleophile to the phosphate group is considered, a concerted mechanism also gives rise to a smaller absolute value of β_{nuc} than β_{lg} (Figure 2e,f). With equation 9 applied to $\Delta\Delta G^{\ddagger}_{2\rightarrow 3}$ one predicts $\beta_{nuc} = 0.4$ and $\beta_{lg} = -0.8$ in the same way as above. With a slightly higher value of $\beta_{2\rightarrow 3} = 0.75$ in equation 9, the calculation exactly reproduces the experimental results [3]. This shows that for a mechanism involving pre-equilibrium PT from a nucleophile that is protonated in the reactant state, there is an intrinsic asymmetry with a larger absolute value of β_{lg} than β_{nuc} that has nothing to do with whether the TS is associative or dissociative. Of course, the dependencies of logk on $pK_a^{nuc/lg}$ in Figure 2a–d cannot be strictly valid over the entire range of $pK_a^{nuc/lg}$, because the TS is bound to be shifted from early to late, or vice versa, at some point. That is, both the associative and dissociative (but not the concerted) mechanisms are expected to show a break in the β_{nuc} and β_{lg} plots when this shift of TS occurs. Experimental data for the dianion hydrolysis reaction mostly cover a limited range of $pK_a^{nuc/lg}$, however, so it is not clear whether such a break exists or not (see Figure 4 of [16] where there is some indication of a change in slope for the monoanion case).

What mechanistic information can then actually be inferred from reported LFERs between phosphoryl transfer reaction rates and nucleophile/leaving group pK_a values? It should be kept in mind that several such reported correlations refer to highly exothermic and asymmetric reactions, such as ester hydrolysis reactions involving the often studied nitrophenyl phosphate leaving groups as well as acyl phosphates and nucleotide monophosphates and diphosphates (AMP, ADP, etc.) [3,16,18,20,30].

Although the attack rates of amines on phosphate monoesters might not be altogether relevant for the processes with oxygen nucleophiles predominantly found in biological reactions, the pioneering study of Kirby and Varvoglis [30] showed a marked insensitivity of reaction rates of 2,4-dinitrophenyl phosphate dianion to a series of substituted pyridine nucleophiles. Yet, the entropy of activation was found to be around −20 entropy units (e.u.) and was therefore interpreted in terms of a bimolecular displacement mechanism [30]. In contrast, a strong dependence on the pK_a for various nitro-substituted phenyl leaving groups was found in reactions with a given nucleophilic agent [30]. Such a comparison should, however, be interpreted carefully because the reactions are clearly 'asymmetric' with respect to the nucleophile/leaving group. For example, semiempirical AM1 calculations on these nucleophiles and leaving groups show that, although the electron density on the leaving oxygen for phenolate groups drops considerably with attached nitro-substitutents, the effect on the nitrogen nucleophile charge caused by nitro- or amide-substituents on pyridines is very small. Nevertheless, similarly large pK_a shifts are observed in both cases. Furthermore, the amine nucleophiles studied by Kirby and Varvoglis [30] cannot involve any pre-equilibrium PT because the pH was adjusted to keep the nucleophile deprotonated (neutral) in the dianion reactions. On the other hand, when the reactions of (protonated) amines with the 2,4-dinitrophenyl phosphate monoanion at low pH was examined a relatively large value of β_{nuc} (0.56) was obtained [30]. It is of interest to note that, for the reaction of 2,4-dinitrophenyl phosphate dianion, the lack of nucleophile sensitivity among a series

of amines is, in fact, contrasted by a more pronounced dependence on different oxygen nucleophiles (compare with Table 5 of [18]). The reaction with H_2O is 340 times slower than with hydroxylamine, whereas OH^-, acetate and F^- attack was not detected at all [18].

There are a few examples of studies of phosphoryl transfer between more symmetric groups, notably those of Skoog and Jencks [31] and Bourne and Williams [32] who examined (PO_3^-) exchange between substituted pyridines. A relatively weak nucleophile/leaving group dependence was found in these cases and the mechanism was interpreted in terms of a more or less symmetric TS with similar bonding to the nucleophile and leaving group. Whether such a transition state or an adjacent high-energy intermediate should be regarded as associative or dissociative would then again depend on the total axial phosphorous bond order change. But, as no proton transfers are involved in this case, the question is merely a matter of bond lengths. It is interesting to note here that no break in the β_{nuc} plot was observed in [31], whereas the reaction rate with isoquinoline as leaving group appears to become independent of nucleophile above a pK_a^{nuc} of ~6 [32]. It does not seem unreasonable to expect the phosphoryl exchange reactions with pyridines (and isoquinoline) to be more concerted than reactions of the hydrolysis type where proton transfers must also take place. It also seems more difficult to envisage a symmetric dissociative dianion transfer transition state (or high-energy intermediate) when the stable form of the free nucleophile and leaving group is protonated, without involving protonation of the phosphate group. Such an alternative is, in principle, equivalent to equation 4 with low axial bond orders in the TS. The low pK_a estimated for metaphosphate, however, makes such a dissociative mechanism seem less likely [19]; this seems to be a reason why the possibility of a pre-equilibrium proton transfer yielding a TS with protonated metaphosphate character is usually excluded in dissociative mechanism proposals. In fact, it is not clear from the literature what the dissociative TS for a symmetrical monoester dianion reaction with axial groups of high pK_a (e.g. simple alcohols) is assumed to look like. In particular, to avoid a protonated metaphosphate-like TS, there must be another reversible mechanism for the net proton exchange between the nucleophile and leaving group.

Another example of LFERs for monoester reactions has been reported by Herschlag and Jencks [33] who measured the reactivity of phosphorylated pyridine monoanions with different oxygen nucleophiles. They find that the relative rate of OH^- attack compared with that of H_2O (k_{OH}/k_{H_2O}) is ~80 times faster for the reaction with phosphorylated pyridine ($pK_a^{lg} = 5.4$). With morpholinopyridine ($pK_a^{lg} = 9$) the corresponding ratio is $k_{OH}/k_{H_2O} = 570$. As noted above, the nature of the leaving group affects the pK_a of the phosphate itself according to $\Delta pK_a(\text{phosphate})/\Delta pK_a(\text{lg}) \sim 0.23$. The pK_a difference between pyridine and morpholinopyridine would then lead to a difference in the phosphate group pK_a of $\Delta pK_a = 0.83$. For a pre-equilibrium proton transfer mechanism, in the case of attack by H_2O (compare with Figure 2b), this pK_a shift yields a PT free energy change of $\Delta\Delta G^0_{PT} = 1.13$ kcal/mol. Such a shift corresponds to the lowering of state (2) in Figure 2b. Measuring the TS barrier height from states (1) and (2), respectively, determines the difference in rate between the H_2O and OH^- nucleophiles (for a given leaving group). The change in phosphate pK due to the leaving groups should therefore be directly reflected in the relative reaction rates k_{OH}/k_{H2O} for a mechanism like equation 2. This can also be seen from Figure 2b in which the relative rate of H_2O and OH^- attack is determined by the free energy difference between states (1) and (2). In fact, with the above value of $\Delta\Delta G^0_{PT}$ we would predict that k_{OH}/k_{H_2O} is seven times larger with $pK_a^{lg} = 9$ than with $pK_a^{lg} = 5.4$, which is exactly the experimentally observed value [32]. It therefore appears again that a mechanism involving pre-equilibrium PT is compatible with experiments.

So, what do the reported LFERs on phosphate-transfer reactions actually tell us? Contrary to claims often found in the literature, we find that they do not generally provide the required information for deciding whether the TS is associative or dissociative. In particular, large negative values of β_{lg} do not prove that the mechanism is a dissociative unimolecular elimination and, in fact, we find that a mechanism with pre-equilibrium proton transfer fits the data equally well, if not better. On the other hand, it appears that for asymmetric reactions of the exothermic type (e.g. hydrolysis of 2,4-dinitrophenyl phosphate) the rate is considerably more sensitive to leaving group than to nucleophile. This could mean that the TS is mainly associated with leaving-group bond fission which can, in principle, occur both early in a dissociative process and late on an associative pathway. A concerted mechanism with pre-equilibrium proton transfer to the phosphate group also seems possible.

Isotope effects

Primary and secondary oxygen kinetic isotope effects in phosphoryl transfer reactions can give information on the change in P–O bonding between the transition and ground states. If a certain P–O bond order decreases in the TS then the ratio between the ^{16}O and ^{18}O isotope reaction rates $^{16}k/^{18}k > 1$ and a normal isotope effect is observed. Conversely, if the bond order increases we get an inverse isotope effect if that particular oxygen is isotopically substituted. Cleland and coworkers have measured secondary ^{18}O nonbridge isotope effects for glucose-6-phosphate hydrolysis in water [34,35] and alkaline phosphatase [13]. In general, as metaphosphate-like transition states might be expected to have an increased bond order to P for the three nonbridge oxygens, one would predict an inverse ^{18}O isotope for such a

mechanism. Conversely, a pentacovalent-like TS should have a decreased bond order to P and a normal isotope effect would then be anticipated. The observed ^{18}O isotope effect for a single nonbridge oxygen was 1.0046 (i.e. normal and not inverse) in the hydrolysis of the glucose-6-phosphate monoanion [35]. This value was then corrected, however, according to the assumption of a pre-equilibrium PT from a nonbridging oxygen to the one bridging the leaving group (equation 3) by the observed equilibrium isotope effect for deprotonation of the phosphate monoanion [34]. Hence, the resulting calculated single ^{18}O isotope effect for P–O bond cleavage of 1.0004 depends on the assumed mechanism rather than proving it. That is to say, for a mechanism like equation 2 the correction would be in the opposite direction and a sizable normal isotope effect would then result from P–O bond cleavage in accordance with an associative mechanism. The situation is also complicated by the fact that *ab initio* calculations on the dissociative mechanism produce a transition state with only partial, rather than complete, proton transfer to the leaving group [23], even when the transfer involves an additional water molecule. Precisely the same arguments as above can, in fact, be made concerning the reported secondary ^{18}O isotope effects in alkaline phosphatase hydrolysis [13]. As the equilibrium nonbridge ^{18}O isotope effect for protonation/deprotonation of phosphate groups is of similar magnitude as that observed for the hydrolytic reaction, the interpretation of the isotope effect on P–O bond cleavage is largely dependent on the assumed mechanism.

In the case of *p*-nitrophenyl phosphate hydrolysis, both ^{18}O bridge and nonbridge, as well as ^{15}N secondary isotope effects have been measured by Hengge *et al.* [36]. When only the dianion of the reactant is involved (pH 10) there can be no proton transfer from the phosphate to the leaving group, and the dissociative mechanism does not require any correction. The experiments in this case show an ^{18}O nonbridge isotope effect of unity, however [36]. Hengge *et al.* [36] suggest an explanation for the absence of the predicted inverse isotope effect in terms of metaphosphate single bonding resonance structures, which might be quite reasonable. An equation-4-type mechanism is again fully compatible with the experiments, however, because a presumably normal isotope effect for formation of a pentacoordinate doubly negative species would be counterbalanced by the inverse $^{18}K_{eq}$ isotope effect on PT between the water nucleophile and the phosphate dianion. The ^{18}O nonbridge isotope effect for the monoanion hydrolysis (pH 3.5) was again found to be greater than unity, rather than inverse, and therefore interpreted as resulting from well-advanced proton transfer to the leaving group [36]. For *p*-nitrophenyl phosphate, the normal ^{18}O bridge isotope effect indicates that the TS indeed involves substantial leaving-group bond fission in accordance with other data (see above). It is therefore very surprising that the corresponding primary isotope effect in alkaline phosphatase is unity. This could well be interpreted as an argument for an associative mechanism in the enzyme.

It therefore appears that the isotope experiments also are subject to interpretation in the framework of either associative or dissociative mechanisms and one has to conclude that they do not provide decisive evidence for distinguishing between the two alternatives. Another issue here, which may obscure the conclusions from nonbridge oxygen isotope effect measurements in particular, is that formal double bonding to P may actually involve significant ionic character [37].

Entropies of activation

The diagnostic value of activation entropies for reaction mechanism elucidation is often difficult to assess. It is generally true that bimolecular nucleophilic substitutions involve a loss of translational entropy for the reactants and, if this were the only entropy factor, one would expect to be able to distinguish purely unimolecular and bimolecular transition states by $\Delta S^{\ddagger}$. There are several other factors that complicate the simple 'translational entropy' picture, however: the possible entropy changes associated with intramolecular degrees of freedom as the transition state is approached; steric factors that determine the configurational volumes available to the reactants during the course of the reaction; substantial solvation/desolvation effects might be associated with charge redistribution and delocalization that will also contribute to $\Delta S^{\ddagger}$; and if proton transfers accompany nucleophilic substitution the energetics of such processes also often contain significant entropy terms.

The data of Kirby and Varvoglis [30] for the reaction of pyridines with 2,4-dinitrophenyl phosphate dianion, monoanion and diester anion shows $\Delta S^{\ddagger}$ values of –20 e.u. A negative activation entropy of this magnitude clearly reflects a bimolecular mechanism (second-order kinetics is also followed [30]). The pyridines studied might, however, be subject to steric hindrance in the TS (especially compared with H_2O, OH^- and so on) so that the actual contribution from loss of translational freedom might well be smaller than 20 e.u. As noted above, the amine nucleophiles studied are also uncharged and do not require deprotonation in the given pH range.

In their study of hydrolysis of phosphate monoesters, on the other hand, Kirby and Varvoglis [16] found substantially more positive entropies of activation. For monoester monoanion hydrolysis $\Delta S^{\ddagger}$ values ranged between –1 and –6 e.u. The value $\Delta S^{\ddagger} = -6.0$ e.u. for 2,4-dinitrophenyl phosphate monoanion can also be compared with that obtained for the dianion, $\Delta S^{\ddagger} = +6.6$ e.u. (see also [39]). The more positive activation entropy for the dianion could, for example, be interpreted as a solvation effect because, whatever the TS may look like, it is bound to

have the –2 charge more delocalized than in the dianion. Such an effect would thus be expected for both associative and dissociative mechanisms. On the other hand, a pre-equilibrium proton transfer between H_2O and a phosphate dianion (eq. 4) is also expected to be associated with a positive entropy contribution (from Table D-174 of [39] one finds $\Delta S^\circ > 0$ in this case). Furthermore, it is possible for both the monoanion and dianion that the H_2O nucleophile is rotationally more restricted in the associated reactant 'complex' (ground state) than in the TS due to H-bonding with the phosphate group. It is also not clear at present exactly how the various force constants involving the phosphate group would change along the associative–dissociative diagonal of Figure 1a. Of interest here is also the observation by Barnard *et al.* [40] that the rate differences for monoanion hydrolysis (e.g., between methyl and *p*-nitrophenyl phosphate) are due to changes in entropy rather than activation energy.

Concerning the overall magnitudes of $\Delta S^\ddagger$ for the hydrolysis of 2,4-dinitrophenyl phosphate (mono- and dianions) compared with the reaction with amines, at least part of the more positive value could come from reduced steric hindrance in the TS, but how much is difficult to say. In this context, it is interesting to note that the (bimolecular) attack of OH^- on CO_2 proceeds with $\Delta S^\ddagger \sim 0$ and that of H_2O with slightly more negative activation entropy [41,42], but far from the –20 e.u. often quoted for bimolecular mechanisms. Regarding the entropic cost of restricting the motion (translational and rotational) of water molecules, Dunitz [43] has reported ΔS° values for incorporation of water into crystalline hydrates. It was found that the entropy cost of immobilizing a water molecule in a crystalline salt is at most 7 e.u. at 300K, and therefore concluded that this would be an upper limit for the cost of binding water to macromolecules [43]. Another estimate of translation entropy loss upon binding that is often used [44], is based on the Sackur–Tetrode equation. This formula, in fact, predicts a standard-state entropy difference between water and pyridine of –4.4 e.u., suggesting that part of the difference in activation entropy between the pyridine and water reaction with 2,4-dinitrophenyl phosphate could simply reflect the difference in translational entropy for the free nucleophiles. In view of above discussion, it appears to us that, although the hydrolysis of phosphate monoesters do proceed with more positive values of $\Delta S^\ddagger$ than the corresponding reactions with amines, the data do not justify postulation of two significantly different mechanisms because the activation entropies can be affected by a number of factors.

Conclusions and prospects

We have examined the often cited evidence for dissociative (unimolecular elimination) solution reactions of phosphate monoesters in more detail. Most of the experimental data used as arguments for such a mechanism are, in fact, subject to interpretations and can equally well be considered consistent with an alternative bimolecular associative or concerted mechanism involving pre-equilibrium proton transfer to the phosphate group. In particular, the often-used argument that a large negative Brønsted coefficient for the rate dependence on leaving group proves the mechanism to be dissociative is fundamentally flawed.

Is there then any way to really determine the nature of the transition states for phosphate monoester reactions? It seems likely that current advances in combining high-level quantum chemical calculations with reliable solvent representations could contribute to solving this type of problem. The interpretation of isotope experiments would also benefit from accurate theoretical calculations that address isotope-substitution effects along the possible reaction pathways. Another important source of information is crystal structures of relevant enzyme complexes with TS analogues, although it is likely that some enzymes can change the mechanisms of their catalyzed reactions. On the other hand, it is indisputable that enzymes do provide a unique negative or complementary picture of the TS of the reaction that takes place in their active site. As far as phosphate monoester reactions are concerned, it is repeatedly found that positive charges ligate the nonbridging oxygens [5–12], which would appear to act anticatalytically in a dissociative mechanism [3,4,12,45]. Moreover, nucleophile–phosphorous–leaving-group distances inferred from bound inhibitors are often incompatible with, at least, the existence of free metaphosphate. It therefore seems quite possible that mechanisms of the associative type can be operational both in enzymes and in solution. It is also possible that the energetics of the associative and dissociative mechanisms are quite similar in solution and that the enzyme active site actually determines which of these mechanisms will be operational. The mechanisms of the phosphate transfer reactions we have discussed here are not yet well established, as one is often led to believe, but rather still require further conclusive determination.

Acknowledgements

J.Å. gratefully acknowledges support from the E.C. Biotechnology Program (DGXII) and from the NFR. A.W. acknowledges support from the NSF (grant MCB-9808638).

References

1. Benkovic, S.J. & Schray, K.J. (1973). Chemical basis of biological phosphoryl transfer. In *The Enzymes.* (Boyer, P.D., ed.), pp. 201–238, Academic Press, New York.
2. Westheimer, F.H. (1981). Monomeric metaphosphates. *Chem. Rev.* **4**, 313–326.
3. Admiraal, S.J. & Herschlag, D. (1995). Mapping the transition state for ATP hydrolysis: implications for enzymatic catalysis. *Chem. Biol.* **2**, 729–739.
4. Maegley, K.A., Admiraal, S.J. & Herschlag, D. (1996). Ras-catalyzed hydrolysis of GTP: a new perspective from model studies. *Proc. Natl Acad. Sci. USA* **93**, 8160–8166.
5. Mildvan, A.S. (1979). The role of metals in enzyme-catalyzed substitutions at each of phosphorous atoms of ATP. *Adv. Enzymol.* **49**, 103–126.
6. Evans, P.R., Farrants, G.W. & Hudson, P.J. (1981). Phosphofructokinase: structure and control. *Phil. Trans. Roy. Soc. Lond. B* **293**, 53–62.

7. Kim, E.E. & Wyckoff, H.W. (1991). Reaction mechanism of alkaline phosphatase based on crystal structures. *J. Mol. Biol.* **218**, 449–464.
8. Lindqvist, Y., Schneider, G. & Wihko, P. (1994). Crystal structures of rat acid phosphatase complexed with the transition-state analogs vanadate and molybdate. *Eur. J. Biochem.* **221**, 139–142.
9. Sondek, J., Lambright, D.G., Noel, J.P., Hamm, H.E. & Sigler, P.B. (1994). GTPase mechanism of G proteins from the 1.7-Å crystal structure of transducin α·GDP·AlF4⁻. *Nature* **372**, 276–279.
10. Klabunde, T., Sträter, N., Frölich, R., Witzel, H., Krebs, B. (1996). Mechanism of Fe(III)-Zn(II) purple acid phosphatase based on crystal structures. *J. Mol. Biol.* **259**, 737–748.
11. Schweins, T., Geyer, M., Kalbitzer, H.R., Wittinghofer, A. & Warshel, A. (1996). Linear free energy relationships in the intrinsic and GTPase activating protein-stimulated guanosine 5′-triphosphate hydrolysis of $p21^{ras}$. *Biochemistry* **35**, 14225–14231.
12. Scheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F.& Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* **277**, 333–338.
13. Weiss, P.M. & Cleland, W.W. (1989). Alkaline phosphatase catalyzes the hydrolysis of glucose 6-phosphate via a dissociative mechanism. *J. Am. Chem. Soc.* **111**, 1928–1929.
14. Butcher, W.W. & Westheimer, F.H. (1955). The lanthanum hydroxide gel promoted hydrolysis of phosphate esters. *J. Am. Chem. Soc.* **77**, 2420–2424.
15. Bunton, C.A., Llewellyn, D.R., Oldham, K.G. & Vernon, C.A. (1958). The reactions of organic phosphates. *J. Chem. Soc.* 3574–3587.
16. Kirby, A.J. & Varvoglis, A.G. (1967). The reactivity of phosphate esters. Monoester hydrolysis. *J. Am. Chem. Soc.* **89**, 415–423.
17. Florian, J. & Warshel, A. (1997). A fundamental assumption about OH^- attack in phosphate hydrolysis is not fully justified. *J. Am. Chem. Soc.* **119**, 5473–5474.
18. Kirby, A.J. & Younas, M. (1970). The reactivity of phosphate esters. Reactions of diesters with nucleophiles. *J. Chem. Soc. (B)*, 1165–1172.
19. Guthrie, J.P. (1977). Hydration and dehydration of phosphoric acid derivatives: free energies of formation of pentacoordinate intermediates for phosphate ester hydrolysis and of monomeric metaphosphate. *J. Am. Chem. Soc.* **99**, 3991–4001.
20. Di Sabato, G. & Jencks, W.P. (1961). Mechanism and catalysis of reactions of acyl phosphatases. *J. Am. Chem. Soc.* **83**, 4400–4405.
21. Gorenstein, D.G., Luxon, B.A. & Findlay, J.B. (1979). Stereoelectronic effects in the reactions of phosphate diesters. *J. Am. Chem. Soc.* **101**, 5869–5875.
22. Yliniemela, A., Uchimaru, T., Tanabe, K. & Taira, K. (1993). Do pentacoordinated oxyphosphorane intermediates always exist? *J. Am. Chem. Soc.* **115**, 3032–3033.
23. Florian, J. & Warshel, A. (1998). Phosphate ester hydrolysis in aqueous solution: associative versus dissociative mechanisms. *J. Phys. Chem.* **102**, 719–734.
24. Florian, J., Åqvist, J. & Warshel, A. (1998). On the reactivity of phosphate monoester dianions in solution: Brønsted linear free-energy relationships do not have a unique mechanistic interpretation. *J. Am. Chem. Soc.*, **120**, 11524-11525.
25. Cramer, C.J. & Truhlar, D.G. (1992). AM1-SM2 and PM3-SM3 parametrized SCF solvation models for free energies in aqueous solution. *J. Comput.-Aided Mol. Design* **6**, 629–666.
26. Warshel, A., Hwang J.K. & Åqvist, J. (1992). Computer simulations of enzymatic reactions: examination of linear free energy relationships and quantum mechanical corrections in the initial proton transfer step of carbonic anhydrase. *Faraday Discuss.* **93**, 225–238.
27. Åqvist, J. & Warshel, A. (1993). Simulation of enzyme reactions using valence bond force fields and other hybrid quantum/classical approaches. *Chem. Rev.* **93**, 2523–2544.
28. Warshel, A., Schweins, T. & Fothergill, M. (1994). Linear free energy relationships in enzymes. Theoretical analysis of the reaction of tyrosyl-tRNA synthetase. *J. Am. Chem. Soc.* **116**, 8437–8442.
29. Bourne, N. & Williams, A. (1984). Effective charge on oxygen in phosphoryl ($-PO_3^{2-}$) group transfer from an oxygen donor. *J. Org. Chem.* **49**, 1200–1204.
30. Kirby, A.J. & Varvoglis, A.G. (1968). The reactivity of phosphate esters: reactions of monoesters with nucleophiles. *J. Chem. Soc. (B)*, 135–141.
31. Skoog, M.T. & Jencks, W.P. (1983). Phosphoryl transfer between pyridines. *J. Am. Chem. Soc.* **105**, 3356–3357.
32. Bourne, N. & Williams, A. (1983). The question of concerted or stepwise mechanisms in phosphoryl group ($-PO_3^{2-}$) transfer to pyridines from isoquinoline-*N*-phosphonate. *J. Am. Chem. Soc.* **105**, 3357–3358.
33. Herschlag, D. & Jencks, W.P. (1989). Evidence that metaphosphate monoanion is not an intermediate in solvolysis reactions in aqueous solution. *J. Am. Chem. Soc.* **111**, 7579–7586.
34. Knight, W.B., Weiss, P.M. & Cleland, W.W. (1986). Determination of equilibrium ^{18}O isotope effects on the deprotonation of phosphate and phosphate esters and the anomeric effect on deprotonation of glucose 6-phosphate. *J. Am. Chem. Soc.* **108**, 2579–2761.
35. Weiss, P.M., Knight, W.B. & Cleland, W.W. (1986). Secondary ^{18}O isotope effects on the hydrolysis of glucose 6-phosphate. *J. Am. Chem. Soc.* **108**, 2761–2762.
36. Hengge, A.C., Edens, W.A. & Elsing, H. (1994). Transition-state structures for phosphoryl-transfer reactions of *p*-nitrophenyl phosphate. *J. Am. Chem. Soc.* **116**, 5045–5049.
37. Horn, H. & Ahlrichs, R. (1990). Energetic measure for the ionic character of bonds. *J. Am. Chem. Soc.* **112**, 2121–2124.
38. Wolfenden, R., Ridgway, C. & Young, G. (1998). Spontaneous hydrolysis of ionized phosphate monoesters and diesters and the proficiencies of phosphatases and phosphodiesterases as catalysts. *J. Am. Chem. Soc.* **120**, 833–834.
39. *CRC Handbook of Chemistry and Physics,* 63rd Ed. (Weast, R.C., ed.), CRC Press, Fl, 1982.
40. Barnard, P.W.C., Bunton, C.A., Kellerman, D., Mhala, M.M., Silver, B., Vernon, C.A. & Welch, V.A. (1966). Reactions of organic phosphates. Part IV. The hydrolysis of aryl phosphates. *J. Chem. Soc (B)* 1966, 227–235.
41. Pinsent, B.R.W., Pearson, L. & Roughton, F.J.W. (1956). The kinetics of combination of carbon dioxide with hydroxide ion. *Trans. Faraday Soc.* **52**, 1512–1520.
42. Magid, E. & Turbeck, B.O. (1968). The rates of the spontaneous hydration of CO_2 and reciprocal reaction in neutral aqueous solutions between 0° and 38°. *Biochim. Biophys. Acta* **165**, 515–524. *6*, 629.
43. Dunitz, J.D. (1994). The entropic cost of bound water in crystals and biomolecules. *Science* **264**, 670.
44. Finckelstein, A.V. & Janin, J. (1989). The price of lost freedom: entropy of biomolecular complex formation. *Protein Eng.* **3**, 1–3.
45. Schweins, T. & Warshel, A. (1996). Mechanistic analysis of linear free energy relationships in p21ras and related systems. *Biochemistry* **35**, 14232-14243.